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# A novel mitochondrial *ATP6* frameshift mutation causing isolated complex V deficiency, ataxia and encephalomyopathy

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ABSTRACT

We describe a novel frameshift mutation in the mitochondrial *ATP6* gene in a 4-year-old girl associated with ataxia, microcephaly, developmental delay and intellectual disability.

A heteroplasmic frameshift mutation in the *MT-ATP6* gene was confirmed in the patient's skeletal muscle and blood. The mutation was not detectable in the mother's DNA extracted from blood or buccal cells. Enzymatic and oxymetric analysis of the mitochondrial respiratory system in the patients' skeletal muscle and skin fibroblasts demonstrated an isolated complex V deficiency. Native PAGE with subsequent immunoblotting for complex V revealed impaired complex V assembly and accumulation of ATPase subcomplexes. Whilst northern blotting confirmed equal presence of ATP8/6 mRNA, metabolic $^{35}$S-labelling of mitochondrial translation products showed a severe depletion of the ATP6 protein together with aberrant translation product accumulation. In conclusion, this novel isolated complex V defect expands the clinical and genetic spectrum of mitochondrial defects of complex V deficiency. Furthermore, this work confirms the benefit of native PAGE as an additional diagnostic method for the identification of OXPHOS defects, as the presence of complex V subcomplexes is associated with pathogenic mutations of mtDNA.



## 1. Introduction

Mitochondrial ATP - ultimately generated by complex V - is almost exclusively the aerobic energy source for all cellular metabolic pathways. The process of ATP production by mitochondrial (mt) ATPase (F1F0-ATP synthase, complex V) is a rotary process driven by an electrochemical proton gradient across the inner mitochondrial membrane.

The human mtATP synthase (mtATPase) is an inner mitochondrial membrane enzyme, consisting of a matrix located F1 catalytic part, which is connected via two stalks with the membrane-embedded F0 part for review see (Jonckheere et al., 2012). The F0 that couples the proton gradient to ATP synthesis by F1 contains among others ATP6 (OMIM #516060) and ATP8 (OMIM #516070), the only two of the 16 subunits of complex V which are encoded by mitochondrial DNA (mtDNA) (Anderson et al., 1981; Wittig and Schaegger, 2008). Complex V deficiency is rare amongst the OXPHOS deficiencies compromising approximately 1% of muscle samples analysed (Jonckheere et al., 2013, 2012; Rodenburg, 2011). Frequent causes of severe mitochondrial encephalomyopathies are due to dysfunction of mtATPase caused by missense mutations in the *MT-ATP6* gene (Table 1). Mutations in ATP6 are a cause of neurogenic muscle weakness, ataxia and retinitis pigmentosa

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(NARP; MIM:551500), Leber hereditary optic neuropathy (LHON; MIM:535000), Leigh syndrome (LS; MIM:256000) and mitochondrial infantile bilateral striatal necrosis (MIBSN; MIM:500003) (Houstek et al., 2004). The most frequent mutations in *MT-ATP6* are m.8993T > G associated with NARP (Debray et al., 2007; López-Gallardo et al., 2014, 2009; Pastores et al., 1994), spastic paraplegia-like disorders caused by m.9176T > G (Kucharczyk et al., 2010; Verny et al., 2011) and m.9185T> C causing sensory neuropathy (Auré et al., 2013; Pitceathly et al., 2012).

Here we describe a patient presenting with ataxia and encephalomyopathy caused by a *de novo* frameshift mutation in *MT-ATP6*.

### 1.1. Patients' medical report

Already in early infancy the girl attracted attention by a sucking weakness and a failure to thrive with a weight gain below the 3rd centile. Her overall motor development was delayed with sitting independently at 15 months and walking at 22 months of age. Often trivial infections caused a setback in her development. The girl showed a tendency to freeze easily. At the age of 4 months, she was diagnosed with gallstones that persisted during follow-up. Diagnostic work-up for known typical etiologies for gallstones remained negative. At the age of 7 months, the girl showed a delay in motor development with symptoms of ataxia. The head circumference was at 4th centile, her length at 43rd centile and her weight at 4th centile. Blood lactate levels were repeatedly slightly elevated (2–3 mmol/l, normal < 1.6 mmol/l). Standard metabolic testing revealed a lactic aciduria with otherwise normal organic acids in urine and an elevated cerebrospinal fluid (CSF) lactate level (3.5 mmol/l; normal range < 2.1 mmol/l). Blood gas analysis, creatine kinase, ammonia, acylcarnitines and amino acids were normal. A cranial MRI at the age of 2 years was normal. Consequently, a skin and muscle biopsy was performed. The girl recovered only very slowly from anaesthesia with extreme weakness that lasted for several days. At last follow-up at the age of 9 years, she shows a mild mental retardation (WISC-IV-IQ 77) and a mild ataxia with a gross and fine motor coordination disorder. Serial ophthalmological examinations showed astigmatism and exophoria but no signs of pigment retinopathy. Several echocardiographic examinations were normal. She is thermosensitive and has difficulties to adapt to extreme temperatures. With hot weather her body temperature rises up to 38 °C and with cold weather falls down to 35 °C. Starting at 4 years of age, she had been supplemented with 3 × 500 mg l-Carnitine per day (ca. 100 mg/kgbw). After four weeks of treatment her daily need of sleep declined remarkably from the previous 16 h to 10–11 h per day. In addition, this supplementation seemed to improve her physical endurance and the dynamics of her movements. Her mother had been a top endurance athlete from the former German Democratic Republic. The family history was unremarkable except for an uncle with mild mental retardation on the maternal side.

## 2. Methods

### 2.1. Molecular genetic analysis

Biochemical results of complex V deficiency prompted sequencing of the mitochondria-encoded *ATP* genes. A C insertion at position m.8612 of the mtDNA (rCRS: NC_012920) was detected by sequencing the *ATP6* gene. Quantification of the heteroplasmic degree of the mutation was performed by amplification of a 554 bp fragment specific to the mtDNA using fwd primer nt.8267–8285 and FAM-labelled rev primer nt.8817-8794. A more convenient amplicon was obtained by *Ssp*I digestion for fragment analysis on an ABI3100 capillary sequencer. Standard RFLP by inclusion of a mismatch in the primer site (Jackson et al., 2014a) was feasible, since the patient also harboured the additional polymorphism m.8610T > C and m.8614T > C resulting in a homopolymeric cytosine stretch. Therefore, an approach by fragment analysis on a capillary sequencer to discriminate the C insertion from wild type sequence was used to calculate the heteroplasmic degree.

### 2.2. Biochemical and microscopic analyses

Primary fibroblast cultures were established from a skin biopsy as described (Schaller et al., 2011b). Light and electron microscopic mitochondrial imaging was performed as described before (Jackson et al., 2014c; Schaller et al., 2011a). Skeletal muscle homogenates were prepared as described previously and the enzymatic activities of the respiratory chain complexes and the mitochondrial matrix marker enzyme citrate synthase were measured

**Table 1**
Confirmed disease-associated *ATP6* mutations in human.

| Position | Amino acid change | Het/hom | Pathology | Reference |
|---|---|---|---|---|
| 8528T > C | M1T $_{\text{W-R (ATP8); M(start)-T (ATP6)}}$ | het | Infantile cardiomyopathy (IC) | Imai et al., 2016 |
| **8611 insC** | **P29LfsX36** | **het** | **ataxia/encephalomyopathy** | **this report** |
| 8618-8619insT | I31fsX63 | het | NARP | López-Gallardo et al., 2009 |
| 8839 G > C | A105P | het | NARP | Blanco-Grau et al., 2013 |
| 8851T > C | W136R | het | BSN | |
| 8969G > A | S148N | het | MLASA | Burrage et al., 2014 |
| 8993T > C/G | L-P/R | het/ho | Leigh disease/NARP/Ataxia/MILS/FBSN/other | deCoo et al., 1996 |
| 9011C > T | A162V | het/ho | Ataxia/AOSA | |
| 9025G > A | G167S | ho | CC, MN, LS-like | López-Gallardo et al., 2014 |
| 9029A > G | H168R | het/ho | LHON-like | López-Gallardo et al., 2014 |
| 9032T > C | L169P | het | NARP | López-Gallardo et al., 2014 |
| 9035T > C/G | p.L170P | het/ho | Ataxia syndromes AOSA | |
| 9176T > G/C | L217R/P | het/ho | LS/Spastic Paraplegia/FBSN | Verny et al., 2011 |
| 9185T > C | L220P | het | Leigh Disease/Ataxia syndromes/NARP-like disease | Kabala et al., 2014 |
| 9191T > C | L222P | het | LS | Kabala et al., 2014 |
| 9205 delAT | Ter-M | het | Seizures/Lactic acidemia | Jesina et al., 2004 |

Adapted from López-Gallardo et al. (2014), Verny et al. (2011), Kabala et al. (2014), Jesina et al. (2004), and Kogelnik et al., (1996). Mutations in *MT-ATP6* (mtDNA NC_012920.1: nt.8527–9207). Mutations associated with LHON: 8668T > C, 8836 A > G, 9016 A > G, 9101T > C, 9139G > A.
Abbreviations HCM Hypertrophic cardiomyopathy, BSN bilateral striatal necrosis, CC colon cancer, LS Leigh syndrome, LHON Leber hereditary opticus neuropathy, MILS Maternally inherited Leigh syndrome, MLASA mitochondrial myopathy, lactic acidosis, and sideroblastic anemia, FBSN familial bilateral striatal necrosis, NARP Neuropathy, ataxia and retinitis pigmentosa, AOSA adult-onset spinocerebellar ataxia.

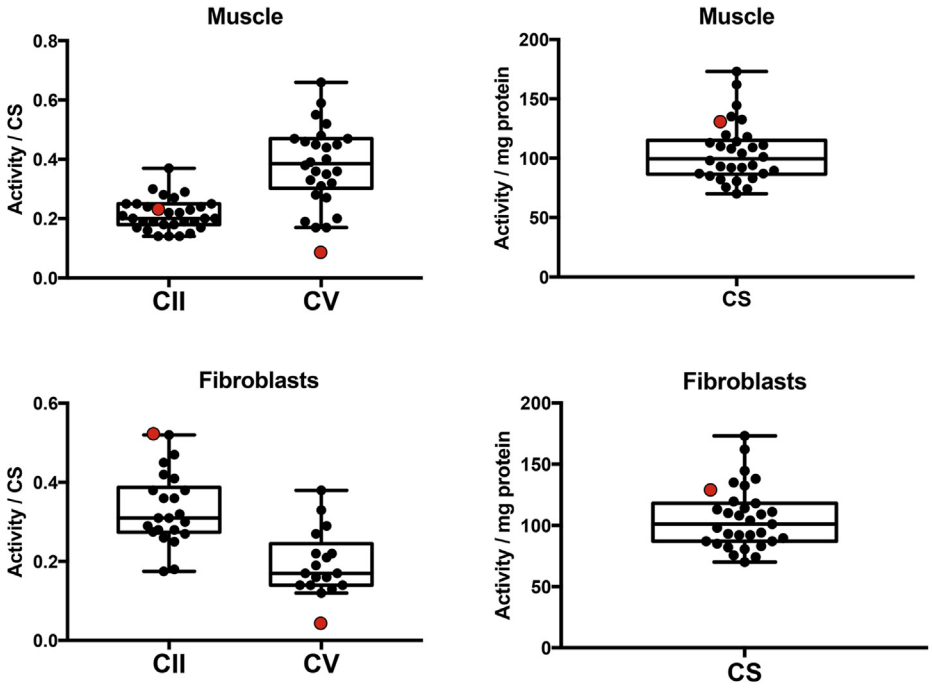


**Fig. 1. Respiratory chain enzyme activities in isolated cultured skin fibroblasts mitochondria and in muscle homogenates.** Both, patient fibroblasts and muscle show an isolated complex V defect with residual activities of 25% and 28% respectively, whilst CS activity was in the higher normal range in fibroblasts and normal in muscle. Red dots represent patient values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spectrophotometrically as described (Jackson et al., 2014b). Isolated mitochondrial proteins from fibroblast were separated by native PAGE as described (Jackson et al., 2014b). Antibodies for western blotting included: β-subunit of complex V (Abcam, ab14730), SDHA (Abcam, ab14715) and ATP6 (Abcam, ab101908). Northern blotting to determine the stability and abundance of the ATP6 transcripts was performed as described (Richter et al., 2013). Briefly, 5 μg of total RNA was run on 1.5% formaldehyde-agarose gels, blotted and probed with a $^{32}$P-ATP-end labelled oligo for RNA14 (ATP8/6 probe: 5′-TGGGTGATGAGGAATAGTGTAAGGAG-3). Radioisotope labelling of mitochondrial translation products was analysed as described before (Richter et al., 2015). Briefly, cells were metabolically labelled with [$^{35}$S]methionine/cysteine (EasyTag; PerkinElmer) for 30 min in the presence of anisomycin to inhibit cytoplasmic translation. Translation products were separated on a 12–20% gradient SDS-PAGE gel, exposed to a phosphor screen and scanned on a TyphoonFLA7000 (GE Healthcare).

## 3. Results

Biochemical measurements of the individual respiratory complexes prompted by the suspicion of a mitochondrial defect were performed from skeletal muscle homogenates and mitochondria enriched fractions of skin fibroblasts. Both tissues showed severe reduction of complex V with a residual activity of 25% in skeletal muscle and 28% in fibroblasts compared to controls (Fig. 1). Microscopic analysis of mitochondria in patient fibroblasts revealed structurally distorted mitochondria with aberrant cristae formation (Fig. 2 A). Mitochondrial protein isolated from patient fibroblasts analysed by western blotting of 1D native PAGE using an antibody against the α-subunit of complex V shows impaired complex V assembly in the patient (Fig. 2 B) and ATP6 protein levels to be drastically reduced (Fig. 2 C). Further electrophoretic analysis of crude mitochondrial fractions from the patient's skeletal muscle by native PAGE (1D BN-PAGE) with subsequent SDS-PAGE (2D-PAGE) confirmed incomplete assembly of complex V in the patient (Fig. 2 D). Complex V subunits were apparent and were identified as ATPase α, ATPase β and F1 subunits. Due to the enzymatically isolated complex V defect and reduced ATP6 protein levels, the mtDNA-encoded subunits of complex V were sequenced (*MT-ATP6/8*) and revealed a heteroplasmic C insertion at position 8611 in the *MT-ATP6* gene (m.8611_8612insC) (Fig. 3 A). Standard RFLP design was impeded by the additional homoplasmic polymorphisms m.8610T > CC and m.8614T > C leading to a 7-mer wild type or 8-mer mutated homopolymer of cytidines with high homology to nuclear background. An mtDNA-specific fragment was amplified and labelled by a last fluorescent cycle (LFC). The 554 bp amplicon was digested using *Ssp*I to obtain a convenient fragment of ~371 bp for SSCP/HD analysis. SSCP analysis revealed heteroduplexes in the patient's skeletal muscle and fibroblasts, which were absent in controls and the mother's blood (Fig. 3B and C). The digested amplicons were analysed on a capillary sequencer and the mutational load of the patient's was calculated to be ~60% in skeletal muscle and ~80% in fibroblasts. The mutation was not detected in the mothers' blood or buccal cells (Fig. 3 A). Subsequent analysis of ATP6 protein levels in isolated mitochondria showed a marked reduction in the patient (Fig. 2 B). Because mutations in mtDNA can affect mRNA stability, we analysed transcript steady state levels of RNA14/15 and RNA14. Northern analysis indicated no change in either precursor RNA14/15 nor in the mature RNA15 steady state abundance (Fig. 4 A). However, assessment of mitochondrial protein translation by metabolic $^{35}$S-labelling shows a marked decrease of newly synthesised ATP6 in patient' fibroblasts and the accumulation of an aberrant translation product (Fig. 4 B).

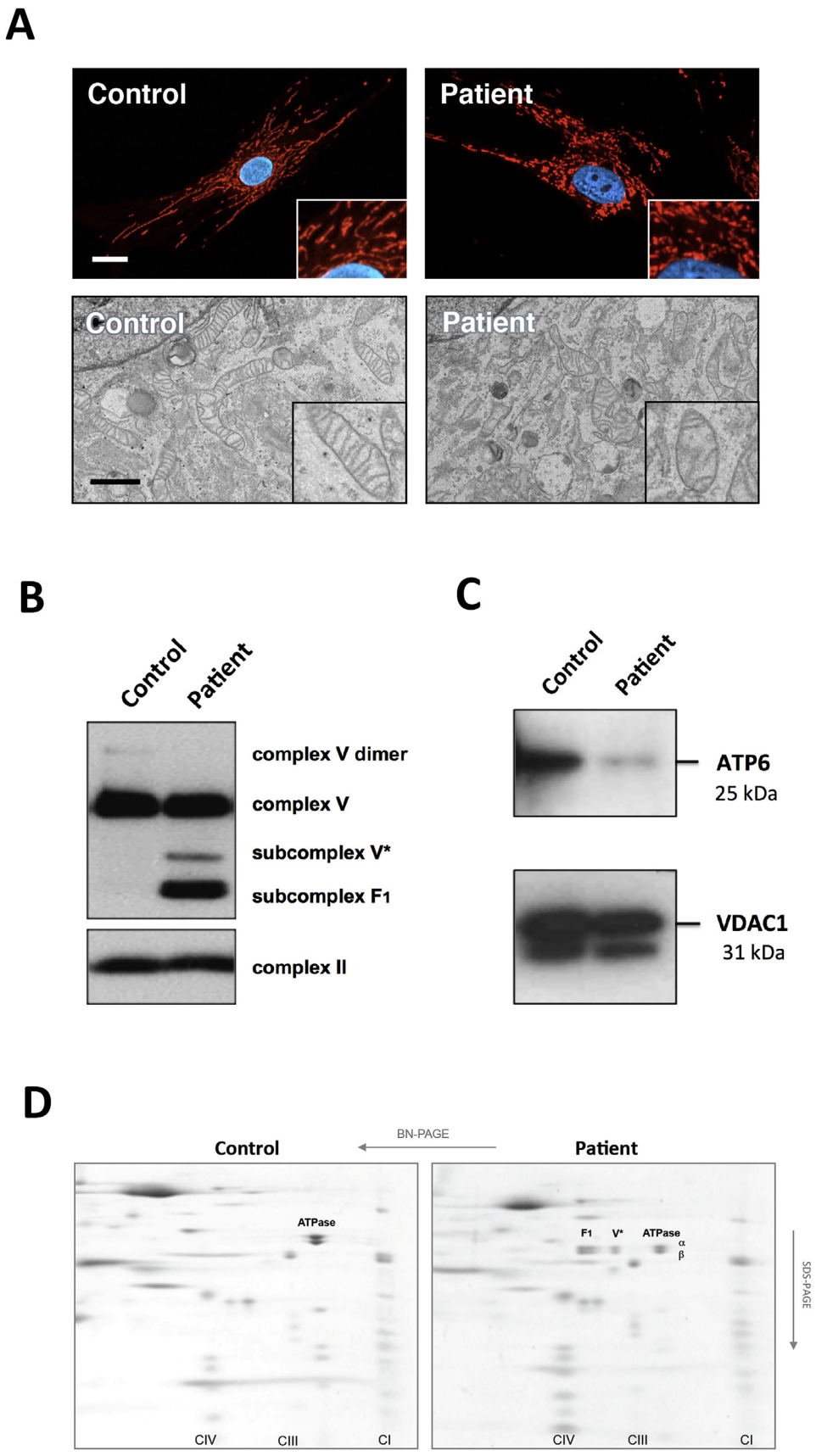


**Fig. 2. Assembly of complex V in skeletal muscle and fibroblasts.** (**A**) Light (upper panel) and electron (lower panel) microscopic analysis reveal distorted mitochondrial shape and disorganised cristae. (**B**) 1D BN-PAGE with subsequent western blotting analysis of skin fibroblasts by an antibody against α-subunit of complex V. Detection of SDHA confirmed equal loading. Patient's fibroblasts showed incomplete assembly of complex V. (**C**) Residual ATP6 protein expression levels in isolated mitochondria of patient fibroblasts. Western blotting with an ATP6 antibody shows marked reduction of steady state levels in the patient compared to the control. VDAC (Porin) is used as a marker of mitochondrial purity and loading. C: control, P: patient. (**D**) After native PAGE, samples prepared from muscle (750 μg) were separated by a second dimension (denaturing 10% Tricine/SDS gel. (2D BN-PAGE) with subsequent coomassie staining. The position of ATPase (CV), V* and F1 subcomplexes in the first dimension and the subunits in the second dimension are indicated. Subcomplexes are annotated from (Carrozzo et al., 2006; Jonckheere et al., 2008).

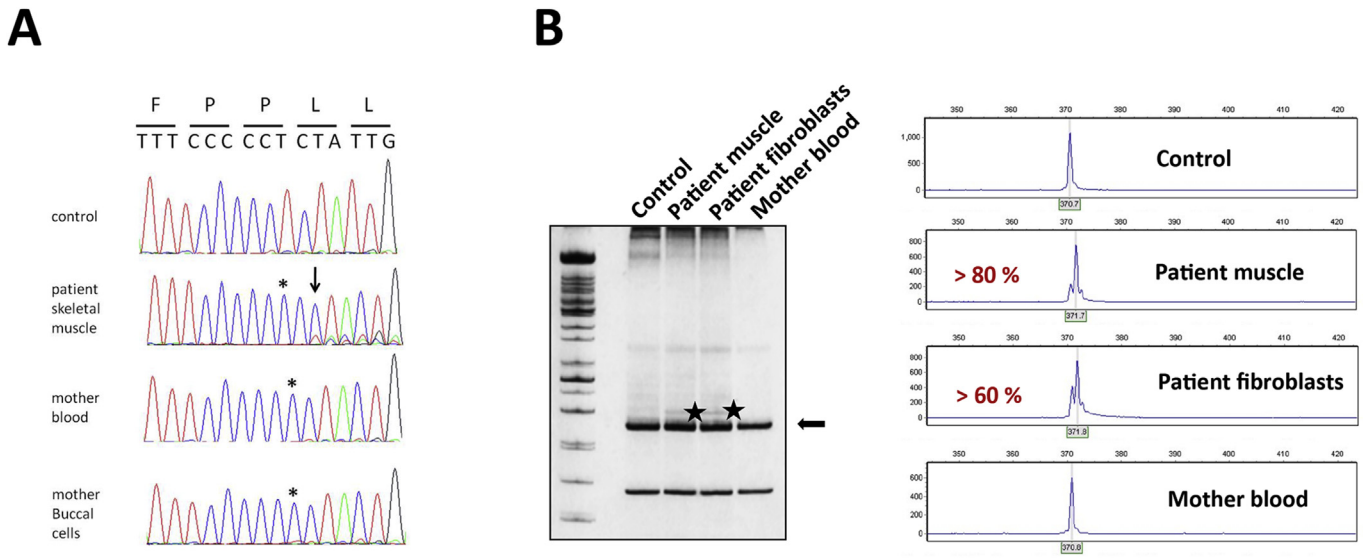


**Fig. 3. Molecular genetic analysis and mutational load quantification by LFC.** (**A**) Sequencing of the *MT-ATP6*-gene revealed a novel heteroplasmic frameshift mutation p. Pro29LeufsX36 (m.8611_8612insC) in DNA extracted from patient skeletal muscle (indicated by an arrow). The mutation was not detected in the DNA extracted from mothers blood or buccal cells. Two additional detected polymorphisms m. 8610 T > C und m.8614 T > C are indicated by asteriks. (**B**) Mutational load quantification by LFC. A mitochondrial-specific PCR product could only be amplified by a fragment of 554 bp. Shorter fragments resulted in unspecific background amplification due to high nuclear homology. Thus the FAM-labelled PCR product was digested with *Ssp*I to obtain a shorter amplicon for improved fragment resolution on the capillary sequencer (left). SSCP/HD analysis detected hetero-duplexes deriving from the mutation in the patient's skeletal muscle and fibroblasts (red asterisk). Analysis of digested fragments detected the single base pair difference due to the C insertion (371 bp and 370 bp) in the patient's skeletal muscle and fibroblasts compared to control and blood of the mother. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

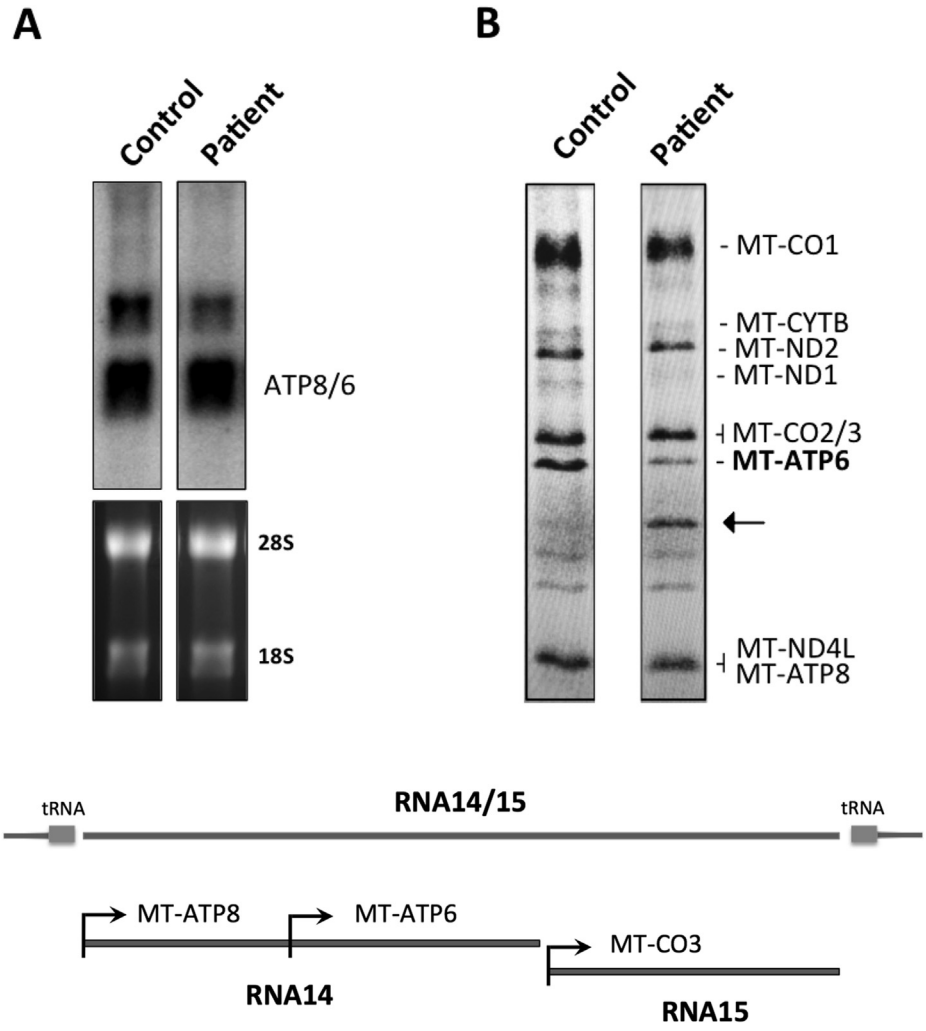


**Fig. 4. Northern blot and metabolic $^{35}$S-labelling for analysis of mitochondrial ATP6 mutant.** (**A**) Steady state transcript analysis by northern blotting for the co-transcript of MT-ATP8 and MT-ATP6 (RNA14). The band above represents the uncleaved RNA14/15 (see schematic) (**B**) Analysis of mitochondrial translation products reveals a drastic decrease in formation of full-length ATP6 protein level compared to healthy control fibroblasts. In addition to this decrease an additional band intensifies, possibly representing aberrant translation product (arrow).

## 4. Discussion

Here we describe a novel *MT-ATP6* frameshift mutation in a patient presenting with ataxia and encephalomyopathy. The C insertion at position m.8611 (m.8611-8612insC) truncates the protein to 36 amino acids (p.Pro29LeufsX36). The novel variant was heteroplasmic with a mutational load of ~60% in skeletal muscle and ~80% in fibroblasts but was absent in the mothers' blood and buccal cells. Biochemical evaluation of individual OXPHOS complexes showed marked complex V deficiency with residual activity of 28% in skeletal muscle and 25% in fibroblasts of the patient compared to controls correlating with the mutational load in both tissues. Protein electrophoretic separation of patient skeletal muscle demonstrated incomplete complex V assembly and revealed accumulated subcomplexes. Incomplete assembly has been shown to be associated with mitochondrial biosynthesis disorders arising from mutations in the mtDNA (Jonckheere et al., 2012) with the mutational load directly correlating with accumulation of subcomplexes of complex V for the NARP mutation m.8993T > C (unpublished results). As the unassembled water soluble F1 part is functional, incomplete ATPase complexes due to mutations affecting ATPase stability are mostly still capable of ATP hydrolysis, but not its synthesis (Dittrich et al., 2003). Additional to the assembly defect in our patient, mitochondrial translation shows a drastic decrease in ATP6 protein production in conjunction with accumulation of aberrant products.

The vast phenotypic spectrum associated with mutations in ATP6 suggests modifying factors. Interestingly, in NARP patients the nuclear background has been shown to extensively modify the deleterious effect of mtDNA mutations in the *MT-ATP6* gene in mutant cells (D'Aurelio et al., 2010).

Alongside and possibly related to its energetic properties, the mitochondrial ATPase functions as structural element by which dimerization of the assembled ATPase complex shapes formation of inner mitochondrial membrane (Davies et al., 2012). In accordance with this, ultrastructural analysis of the fibroblasts of the patient reported here revealed abberrant cristae formation. In summary, biochemical analyses reveal incomplete assembly of complex V and reduced activity in skeletal muscle and fibroblasts, distorted mitochondrial ultrastructure and aberrant translation in our patient. In conclusion, these results corroborate the pathogenicity of this novel heteroplasmic mutation and correlate with the disease phenotype. Intriguingly, supplementation of L-carnitine drastically improved the patient's ataxia symptoms, the need for sleep and furthermore the adaptation to temperature extremes. Whether this supplementation was causal for her improvement is uncertain. L-carnitine supplementation effects on the ATP production in tissues dependent on β-oxidation such as skeletal muscle by elevation of free carnitines and thus acts to deplete toxic acyl compounds (Dimauro et al., 2006; Parikh et al., 2009).

This report describes a frameshift mutation in the *MT-ATP6* gene causing impaired complex V assembly and deficiency in a patient presenting with ataxia and encephalomyopathy.

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## Competing interests

No conflicts of interest to declare.

## Ethical approval

Parental consent has been obtained and the study was approved by the local ethical committee of the Canton of Berne.

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